

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
21 April 2005 (21.04.2005)

PCT

(10) International Publication Number
WO 2005/035782 A1

- (51) International Patent Classification⁷: **C12Q 1/18**, G01N 33/50 (74) Agent: WINCKELS, J., H., F.; Vereenigde, Johan de Wit-
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- (21) International Application Number: PCT/NL2004/000719 (81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD,
MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG,
PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM,
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM,
ZW.
- (22) International Filing Date: 13 October 2004 (13.10.2004)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 03078246.0 13 October 2003 (13.10.2003) EP (84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI,
SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, ML, MR, NE, SN, TD, TG).
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- Published:
— with international search report
- For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: METHOD OF CLASSIFYING CHEMICAL AGENTS AND IDENTIFYING CELLULAR TARGETS THEREOF

(57) Abstract: The present invention is in the field of characterization of antibiotic compounds, e.g. such as compounds useful for treating pathogenic infections in mammals and identification of cellular targets thereof. The invention further relates to a method of classifying antibiotic compounds, to methods for determining their modes of action and for identifying new antibiotic targets based on classification schemes comprising clustering according to similarity the biochemical response profiles (e.g. gene expression pattern) of an organism exposed to said compounds, for a period of less than one generation time.

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Title: Method of classifying chemical agents and identifying cellular targets thereof.

FIELD OF THE INVENTION

The present invention is in the field of characterization of antibiotic compounds, e.g. such as compounds useful for treating pathogenic infections in mammals and identification of cellular targets thereof. The invention further relates to a method of classifying antibiotic compounds and to methods for determining their modes of action.

BACKGROUND OF THE INVENTION

Outbreaks of foodborne and waterborne infectious disease are on the increase around the world and are a serious public health threat. To make matters worse, the percentage of micro-organisms that are resistant to antibiotics is also increasing rapidly, and researchers are becoming increasingly concerned about crossover resistance associated with the food we eat and the water we drink. Of specific concern is resistance to methicillin in strains of *Staphylococcus aureus* (MRSA) and resistance to the antibiotic of "last-resort" vancomycin in strains of Enterococcus bacteria (VRE) and the spread thereof to other organisms.

In the past, the solution to bacterial resistance has been primarily dependent on the development of clinically viable anti-microbial agents. Apart from the discovery of natural antibacterial peptides from plants and animals, there have been few new antibiotics developed in recent years. As a result, little is presently expected of the traditional antibiotic screening methods, based on direct measurements in living cells of the inhibitory capacities of particular compounds.

In light of the foregoing it would be a significant advancement in the art to provide a method of identifying the molecular target of a lead compound, i.e.

an antibiotic compound that is non-toxic to humans. It would be a further advancement if the method was capable of identifying new molecular targets for antibiotic compounds.

5 SUMMARY OF THE INVENTION

The present invention is based on the insight that intrinsic changes in the biochemical composition of a micro-organism which result from external stimuli received by that micro-organism from its surrounding environment are characteristic for the nature of the stimulus. Therefore, by measuring these
10 intrinsic changes, the micro-organism may be used as a sensor to determine the condition of its environment and, more specifically, the nature of the stimulus. An example of such a stimulus is a chemical substance that is capable of inhibiting the growth or even of killing a micro-organism, such as an antibiotic compound. It has now surprisingly been found that the reaction of a
15 micro-organism to substance exposure and the specific way in which that substance affects the growth of the micro-organism can be used to classify those substances, e.g. either by mode of action of the substance, by molecular target of the substance and/or by metabolic route affected by the substance.

The present invention now utilizes this finding to provide, in a first
20 aspect, a method of producing a classification scheme for chemical compounds comprising clustering according to similarity the biochemical response profiles of an organism as determined by exposing said organism for a of period less than one generation time to a multitude of individual chemical compounds. In a preferred embodiment, said clustering according to similarity comprises
25 principal component analysis. In another preferred embodiment said chemical compounds are antibiotic compounds. In yet another preferred embodiment said multitude of individual chemical compounds comprises reference compounds with known mode of action and wherein said profiles are reference response profiles clustered according to similarity in mode of action.

In another aspect, the present invention provides a classification scheme for chemical compounds obtainable by a method of the invention.

In a further aspect the present invention provides a method of classifying a chemical compound, comprising the steps of providing a classification scheme for chemical compounds by performing a method of the invention of producing a classification scheme for chemical compounds as described above, exposing an organism to said chemical compound, determining the biochemical response profile of said organism, and determining in said scheme the clustered position of said biochemical response profile.

In yet a further aspect the present invention provides a method of classifying an antibiotic compound according to mode of action comprising the steps of providing a classification scheme for chemical compounds by performing a method of producing a classification scheme for chemical compounds as described above, wherein the chemical compound is an antibiotic compound, wherein the multitude of individual antibiotic compounds comprises reference compounds with known mode of action and wherein the profiles are reference response profiles clustered according similarity in mode of action, said method comprising the further steps of exposing an organism to said antibiotic compound, determining the biochemical response profile of said organism, determining in said scheme the clustered position of said biochemical response profile, and assigning to said antibiotic compound the mode of action of the corresponding cluster of reference response profiles or assigning to said antibiotic compound a new mode of action in case said position is outside any known cluster of reference response profiles.

In yet a further aspect, the present invention provides a method of analysing a mode of action of an antibiotic compound comprising the steps of performing a method of classifying an antibiotic compound according to the invention, wherein the biochemical response profile is a transcription response profile, thereby determining for said antibiotic compound the clustered

position of the transcription response profile, identifying from said transcription response profile the genes of said organism of which the expression is substantially affected by exposure to said antibiotic compound and which characterize said clustered position, and determining from the identity of said genes the cellular process affected by said antibiotic compound.

In yet a further aspect the present invention provides a method of identifying a new mode of action of an antibiotic compound comprising the steps of performing a method of classifying an antibiotic compound according to the invention, wherein the biochemical response profile is a transcription response profile, selecting an antibiotic compound of which the corresponding transcription response profile has a clustered position outside any known cluster of reference response profiles, identifying from the transcription response profile of said selected compound the genes of said organism of which the expression is substantially affected by exposure to said selected antibiotic compound and which characterize said clustered position, and determining from the identity of said genes the cellular process affected by said selected antibiotic compound thereby identifying the new mode of action.

In yet a further aspect the present invention provides a method of identifying the molecular target of an antibiotic compound comprising the steps of performing a method of analysing a mode of action of an antibiotic compound as described above or performing a method of identifying a new mode of action of an antibiotic compound as described above, selecting a group of potential molecular targets characterizing the cellular process affected by the antibiotic compound, and identifying the molecular target of the antibiotic compound in the group of potential molecular targets.

In yet a further aspect the present invention provides a method of identifying a new metabolic route comprising the steps of performing the method of classifying a chemical compound as described above, wherein the biochemical response profile is a transcription response profile, wherein said multitude of individual chemical compounds comprises reference compounds

with known mode of action and wherein said profiles are reference response profiles clustered according similarity in mode of action, selecting a chemical compound of which the corresponding transcription response profile has a clustered position outside any known cluster of reference response profiles, identifying from said transcription response profile the genes of said organism of which the expression is substantially affected by exposure to said chemical compound and which characterize said clustered position, and determining from the identity of said genes the cellular process affected by said chemical compound thereby identifying a new metabolic route.

DESCRIPTION OF THE FIGURES

Figure 1 shows a flow chart of the method of producing a classification scheme for chemical compounds essentially as claimed in claim 1.

Figure 2 shows a flow chart of the method of classifying a chemical compound essentially as claimed in claim 9.

Figure 3 shows a flow chart of the method of classifying an antibiotic compound according to mode of action essentially as claimed in claim 10.

Figure 4 shows a shows a score plot in which the data from the experiments described in Example 1 is depicted.

Figure 5 shows a shows a score plot in which the data from the experiments described in Example 2 is depicted.

DETAILED DESCRIPTION OF THE INVENTION

The term "item" as used herein refers to anything classifiable by a method of the present invention and includes both a chemical compound, e.g. an antibiotic compound, and a biochemical response profile, e.g. a transcription response profile or a reference profile.

The term "generation time" as used herein refers to the constant time interval of each cell division during log phase of growth of a micro-organism

(i.e. the doubling time [t_d]) which may be calculated from $t_d = \ln 2 / \mu$, wherein μ is the specific growth rate. The log phase is the period of maximum growth when there is a linear relationship between the log of cell number (or the log of cell mass) and time, which linear relationship can be described as: $dN/dt = \mu N$ or $dx/dt = \mu x$, where N = cell number, x = cell mass (g), t = time (h), and μ = specific growth rate (h⁻¹). A number of factors can influence the generation time of cells: a) The physical growth conditions. Cells grow faster under optimal conditions than under sub-optimal conditions. For example, in *Escherichia coli* the t_d at 15°C is 180 minutes, while at 37°C it is only 20 minutes (assuming all other conditions are optimal); b) The nutritional growth conditions. Cells grow faster in a nutritionally rich medium than in a poor one. For example, in *E. coli* the t_d in nutrient broth approximates 20 minutes and in a chemically defined medium is may be as much as 50 minutes (assuming all other conditions are optimal); c) The type of organism. Some species inherently grow faster than others. For example, under their respective optimal conditions *E. coli* has a t_d of 20 minutes while *Lactobacillus casei* has a t_d of 60 minutes.

The term "classifying" is used in its art-recognized meaning and thus refers to arranging or ordering items, i.e. chemical compounds or response profiles, by classes or categories or dividing them into logically hierarchical classes, subclasses, and sub-subclasses based on the characteristics they have in common and/or that distinguish them. In particular "classifying" refers to assigning, to a class or kind, an unclassified chemical compound or response profile. A "class" then being a grouping of items, based on one or more characteristics, attributes, properties, qualities, effects, parameters, etc., which they have in common, for the purpose of classifying them according to an established system or scheme.

The term "classification scheme" is used in its art-recognized meaning and thus refers to a list of classes arranged according to a set of pre-

established principles, for the purpose of organizing items in a collection or into groups based on their similarities and differences.

The term "clustering" refers to the activity of collecting, assembling or uniting into a cluster or clusters items with the same or similar elements, a
5 "cluster" referring to a group or number of the same or similar items gathered or occurring closely together. "Clustered" indicates that an item has been subjected to clustering.

The term "clustered position" refers to the location of an individual item in amongst a number of clusters, said location being determined by clustering
10 said item.

The process of clustering used in a method of to the present invention may be any mathematical process known to compare items for similarity in characteristics, attributes, properties, qualities, effects, , etc., through data from measurable parameters. Statistical analysis, such as for instance
15 multivariate analysis, or other methods of analysis may be used. Preferably methods of analysis such as self-organising maps, hierarchical clustering, multidimensional scaling, principle component analysis, supervised learning, k-nearest neighbours, support vector machines, discriminant analyse and/or partial least square methods are used. In a most preferred embodiment of a
20 method of the present invention principal component analysis is used to cluster items according to similarity.

A "biochemical response profile" as used herein refers to a set of data, usually in graph or table form, that represents the qualitative or quantitative change in the biochemical composition of an organism resulting from a
25 stimulus, *i.e.* an exposure of that organism to a chemical compound.

Throughout this description with said biochemical composition is meant the composition of the various (bio)chemical compounds that together form the cell and with said change in the biochemical composition is meant the composition before the stimulus minus the composition after the stimulus, corrected for
30 general test influences.

A "transcription response profile" as used herein refers to a set of data, usually in graph or table form, that represents the qualitative or quantitative change in the mRNA pool of an organism resulting from a stimulus, *i.e.* an exposure of that organism to a chemical compound. A "transcription response
5 profile" may suitably be obtained by transcription profiling, the method that uses 10 to thousands of different DNA probes arranged in microarrays, with each DNA representing a different gene or part of a gene, to simultaneously quantify all or a large proportion of the different mRNA species present in the cell at a certain point in time thereby characterizing the transcriptome of that
10 cell. The term "response profile" indicating the composition of mRNA species before the stimulus minus the composition of mRNA species after the stimulus, corrected for general test influences.

In principle, any organism may be used in the methods of the present invention including plants, animals (including human) and preferably micro-organisms. Micro-organisms useful in methods of the present invention may
15 include bacteria, archaea, fungi, yeast, protozoa and algae. More preferably bacteria, fungi and/or, yeast are used.

A "susceptible" organism in the context of the present description is an organism that is responsive to a chemical compound, *i.e.* that shows a
20 detectable change in its chemical composition after exposure to said chemical compound. Thus, "susceptible" not only relates to growth inhibition by antibiotics, but is used herein in a more generalized context.

A "reference compound" is a chemical compound of which one or more characteristic used for classifying chemical compounds is known and that is
25 used to establish reference response profiles. Known characteristics may include for instance the mode of action and/or target in case the compound is an antibiotic.

Suitable reference compounds include such antibiotics as erythromycin, tetracyclin and chloramphenicol as inhibitors of protein synthesis, colistin and
30 polymyxin B sulphate as compounds interfering with membrane integrity,

ciprofloxacin and rifampicin as compounds interfering with nucleic acid synthesis and ceftazidin and ampicillin as compounds interfering with cell wall synthesis.

The term "similarity" indicates the state or quality of being similar, i.e. of having the same or some of the same characteristics, such as mode of action, target, or biochemical response profile.

A known cluster of reference response profiles is a cluster of which the mode of action, target or cellular process affected is known. Such knowledge may be acquired from literature data or from empirical testing or from performing the methods of the present invention.

The term "affected" includes such meanings as acted upon, influenced, or altered, transformed, modified and/or changed. In the context of gene expression it may in particular indicate either an increase or decrease therein, whereas in the context of a cellular process it may in particular indicate an inhibition or stimulation thereof.

A "molecular target" is the cellular component of which the function is disturbed by the action of an antibiotic compound. Molecular targets known to the art include the peptidoglycan bridge in cell walls, the protein synthesis machinery in the form of the ribosome, and many proteins such as RNase P, dihydrofolate reductase and DNA gyrase. A mode of action of an antibiotic is for instance interference with protein synthesis, interference with membrane integrity or cell wall synthesis.

In general, the prior art approach of identifying targets of antibiotics is characterized by first identifying genes whose expression is affected and inferring from the thus obtained genetic information the possible targets or modes of action.

Conversely, the approach of the present invention is characterized by producing a large number of biochemical reference response profiles in combination with known modes of action, known targets and/or known metabolic routes affected, then analysing those profiles for common

characteristics, i.e. clustering said profiles, and once a unique profile for a certain compound is found amongst the known clusters investigating said profile to yield the genetic information required for establishing e.g. the new metabolic route or new target. This approach is much more efficient and cost effective than the methods of the prior art.

A method of the present invention comprises the selection of potential antibiotic compounds by using classical microbiological techniques of testing growth inhibition of a target organism by a chemical or biological compound. Preferably said compound tests negative in a cytotoxicity test in mammalian cells and may thus potentially be used as an antibiotic. Growth inhibition of the organism indicates the susceptibility of the organism to the compound.

The selected potentially antibiotic compounds are then applied to generate biochemical response profiles as follows: The target organism is exposed to a high dose (preferably higher than the Minimum Inhibitory Concentration or MIC) of the test compound, preferably in a mid log culture of said organism. After exposure for a duration of between 1 second and 24 hours, preferably between 1 and 20 minutes, the cellular metabolism in the cells of the organism is fixed or quenched. Quenching may suitably occur by using by immediately quenching the cellular metabolism with 4 volumes of 60% methanol at -40°C. Subsequently, the biochemical composition of the organism is determined. For instance, RNA molecules are isolated and labeled fluorescently in order to be detected by DNA micro-array hybridization. Said DNA micro-array representing for instance the genome of the organism. The hybridization results, i.e. in this case the transcription profiles, obtained with exposed cells are compared to the hybridization results obtained with normal, non-exposed cells that were cultured under identical conditions to provide transcription response profiles.

It is a feature of the present invention that the exposure to the chemical compound is short, and preferably at a concentration higher than the Minimum Inhibitory Concentration. With "short" is meant that the exposure is

such that the organism is essentially not allowed to undergo the complete growth cycle in the presence of said compound. Instead, it is preferred that the effects on gene expression are limited to those effects that emanate directly from the exposure to the chemical compound and not from pleiotropic effects, such as cell death or effects indirectly associated with reduced growth or growth arrest. In general, a suitable duration of the exposure to the chemical compound may be expressed as being less than one generation time, more preferably a time in the range of about 1 to about 45 minutes, even more preferably a time in the range of about 1 to about 15 minutes, most preferably a time of about ten minutes. The skilled person will understand that, in order to measure the true effects of the chemical compound on the micro-organism, the time of the exposure may differ between compounds, since some compounds will effect membrane integrity, resulting in an almost immediate cascade of cellular reactions, whereas other compounds will have an effect on protein synthesis, resulting in a more gradual reaction on the metabolism, e.g. on the expression profile. Likewise, the time of the exposure may differ between organisms and between different cultivation conditions.

It is a preferred feature of the present invention that the cellular metabolism is quenched. This means that the arrest of the cellular metabolism is essentially immediate. Many of the prior art techniques suffer from exposure regimes that are uncontrolled or undefined, because the metabolism is not instantly stopped or arrested since no quenching is performed as a result of which the effects of altered expression observed may be considerably pleiotropic.

The transcription response profiles obtained after exposure of a susceptible organism to different compounds are preferably compared by principal component analysis which then provides for the clustering of transcription profiles according to e.g. similarity in biological mode of action or other similar parameters. The classification of compounds based on e.g. their mode of action is possible using the clusters thus obtained.

This classification of compounds is central to the approach of the present invention in that from this classification a multitude of possibilities arises. For instance, various classification schemes may be generated that are for instance based on the knowledge available on reference compounds such as knowledge on targets or modes of action or cellular processes affected. Alternatively, classification may be based purely on clustering of response profiles.

Based on one of the above methods of classifying, a compound may be selected for further analysis. This analysis may comprise in a first instance the selection and identification of those genes whose expression is strongly affected in a certain cluster of transcription profiles. Based on these results it is determined which biological process in the cell is influenced by the compound. This information then forms the basis for selecting a limited group of potential molecular targets for the compound concerned.

In the search for new antimicrobial strategies, the classification of antibiotic compounds according to the present invention now facilitates identification of biochemical response profiles that are dissimilar to any known response profiles available in e.g. a database. From this, it is inferred that the response profile obtained is new and that either the target of the compound is new, or that the mode of action of the compound is new. Thus, by using the methods of the present invention one is able to search for new antibiotic compounds and/or to search for new targets.

Moreover, the present invention allows for the detection of suitable antimicrobial compounds for organisms that are not extensively studied. This is in contrast to methods of the prior art, wherein a substantial part of the function of the genome of the micro-organism of use is already known (often used is e.g. *Saccharomyces*). Such prior art methods merely allow for the identification of the metabolic routes that are affected by a known compound. This feature of the present invention therefore is that it allows the specific detection of antimicrobial compounds that may be used to influence the growth of newly found strains of micro-organisms without an in-depth knowledge of

the genome. Thus, it is possible with the present invention to identify small-spectrum antibiotic compounds, i.e. compounds that are capable of arresting the growth of only a specific micro-organism, preferably a specific strain. It can easily be seen that such a new antibiotic compound would be very useful in a clinical setting. First of all, it would be specific for the strain which causes the infectious disease, without tampering with all other micro-organisms in the body, which often are useful (intestinal flora). Further, such an antibiotic compound will not be able to cause cross-tolerance, which reduces the risk of generating multi-resistant bacterial strains.

10 In practice the biochemical composition of an organism is the collection of measured data from biomolecules of organisms, preferably of micro-organisms, used in a method of the present invention. Said measured data are preferably obtained from qualitative measurements, but (semi)quantitative measurements may also be used.

15 A suitable collection of measured data is formed by the transcriptional expression profile of an organism, which may for instance be obtained by measuring the various messenger RNA molecules in the cell, i.e. the transcriptome. An alternative collection of measured data is formed by the protein expression profile of an organism obtainable by measuring the various protein molecules present in the cell, i.e. the proteome. Yet another alternative is the collection of metabolites, i.e. the metabolome.

The biochemical composition of an organism, which mirrors the biological condition or state of a cell, may thus suitably be measured by measuring the transcriptional state, whereby amounts of RNA are determined, the translational state, whereby amounts of protein are determined, the activity state, whereby enzymatic activities are determined, or the state of metabolic routes, whereby amounts of metabolites are determined, or by measuring combinations thereof. In fact, measurements of many different biomolecules may provide a biochemical composition of an organism that may be used in a methods of the present invention.

Such biomolecules may comprise polynucleotides, such as nucleic acids, (poly)peptides or proteins, polysaccharides, lipids, lipopolysaccharides and/or other cellular macro molecules. Also metabolic intermediates such as sugars, organic acids, alcohols, fatty acids, amino acids, nucleotides, and the like may
5 be measured. In preferred embodiments of aspects of the present invention nucleic acids, such as RNA, including messenger, transfer and ribosomal RNA or combinations thereof are measured.

In more preferred embodiments of the methods of the present invention the biochemical composition of an organism is determined by determining the
10 transcriptional state of the cell, i.e. as measured in the form of messenger RNA.

Measurements of biomolecules of an organism that may be used for creating a collection of measured data that together provide a biochemical composition are preferably performed in the form of a parallel biochemical
15 analysis method. Such measurements may for instance be performed by separating the cellular biomolecules, labeling them collectively or individually and detecting them qualitatively or quantitatively.

In this context, with separating the cellular biomolecules is meant the physical separation of groups of identical biomolecules on the basis of their
20 specific biochemical features, with the aim of detecting and, if necessary quantifying the thus separated biomolecules. Physically separating the various groups of identical biomolecules is, however, not always necessary. In this context, with labeling of biomolecules is meant the specific or a-specific labeling of biomolecules with specific markers, with the aim of detecting and, if
25 necessary quantifying the thus labeled biomolecules. Such techniques are well known to the art.

In order to perform such measurements use may be made of one or more techniques such as:

- *in situ* measurement techniques such as nucleic acid probe
30 technology and/or immunological measurement techniques; using these

techniques, various cellular constituents may be measured separately or individually, without factual separation thereof by using specific or a-specific detection techniques, the suitability of which depends on the substance to be detected;

5 - electrophoretic and/or chromatographic measurement techniques; using these techniques cellular constituents may be separated on the basis of *i.a.* size, weight, charge, sensitivity to denaturation, after which detection may occur by using specific or a-specific detection techniques, the suitability of which depends on the substance to be detected;

10 - micro array and/or DNA biochip techniques; using these techniques biochemical constituents are separated on the basis of affinity for a surface-immobilised binding partner whereafter detection may occur by using specific or a-specific detection techniques, the suitability of which depends on the substance to be detected.

15 Suitable detection techniques that may be applied in concert with the above techniques include autoradiographic detection techniques, detection techniques based on fluorescence, luminescence or phosphorescence or chromogenic detection techniques. These detection techniques are known in the art of detection of biomolecules.

20 Preferably in a method of the present invention the transcriptional state of an organism is measured by using techniques that involve hybridization to arrays of nucleic acid probes or nucleic acid mimicking probes.

 In order to determine the transcriptional state of an organism the RNA, including both total RNA and mRNA, may be isolated from one or more cells of said organism. Every RNA isolation method capable of isolating mRNA may be
25 used for such isolation procedure (see for instance Ausubel *et al.*, 1987-1993, *Current Protocols in Molecular Biology*, John Wiley & Sons. Inc. New York). Large amounts of samples may be treated by using methods known to the skilled person, such as the single-step isolation-process of Chomczynski (1989,
30 U.S. Pat. No. 4,843,155).

Transcripts (the RNA copies that are the initial step in decoding the genetic information) represent RNA of differentially expressed genes, and may be identified in various samples by using a variety of methods known to the skilled person. For instance, differential screening (Tedder *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:208-212), subtractive hybridization (Hedrick *et al.*, 1984, Nature 308:149-153; Lee *et al.*, 1984, Proc. Natl. Acad. Sci. USA 88:2825), differential display (Liang & Pardee, 1992, Science 257:967-971; U.S. Pat. No. 5,262,311) expressed sequence tag (EST) (Adams *et al.*, 1991, Science 252:1656), serial analysis of gene expression (SAGE) (Kinzler *et al.*, U.S. Pat. No. 5,695,937) or cDNA AFLP (Vos *et al.*, 1995, Nucleic Acids Res., 23, 4407-14) techniques may be used.

The identification of such differentially expressed genes may then be used to design specific markers or labels that can be used to determine in an expeditious manner the presence of particular (levels of) mRNA species that indicate the type of stimulus that the organism receives.

In order to measure the various mRNA molecules in an organism the mRNA is preferably first converted into complementary DNA (cDNA), for which known techniques are at the disposal of the skilled person, such as for instance reverse transcription of the mRNA into cDNA by using the enzyme reverse transcriptase. Optionally, the conversion of RNA into cDNA may be combined with amplification of the cDNA by PCR (Mullis 1987, U.S. Pat. No. 4,683,195, 4,683,202, en 4,800,159) – optionally in a nested, multiplex or asymmetrical format – or by using the ligase chain reaction (Barany 1991, Proc. Natl. Acad. Sci. USA 88:189-193; EP Application No., 320,308), self sustained sequence replication (3SR) (Guatelli *et al.*, 1990, Proc. Natl. Acad. Sci. USA 87:1874-1878), strand displacement amplification (SDA)" (U.S. Pat. Nos. 5,270,184, and 5,455,166), transcriptional amplification system (Kwoh *et al.*, Proc. Natl. Acad. Sci. USA 86:1173-1177), Q-Beta Replicase (Lizardi *et al.*, 1988, Bio/Technology 6:1197), Rolling Circle Amplification (RCA) or any other method for amplifying nucleic acids. In an alternative method, the RNA may

also be directly used or be amplified prior to use by using available RNA amplification methods such as Nucleic Acid Sequence-Based Amplification (NASBA) (L. Malek *et al.*, 1994, Meth. Molec. Biol. 28, Ch. 36, Isaac PG, ed., Humana Press, Inc., Totowa, N.J.) or Transcription Mediated Amplification (TMA).

Methods for obtaining genetic information by using nuclei acid arrays are well known in the art (see i.a. Chee *et al.*, 1996, Science 274(5287):610-614).

In a method according to the present invention, the application of a DNA microarray is preferred. Such arrays of oligonucleotides comprise for instance DNA sequences that are specific for certain mRNA species or for genetic markers. In particular, it is preferred that the array is comprised not only of binding partners for functional genetic units, such as tentative (predicted) open reading frames (ORFs) as identified after functional genomic analysis. Rather, it is preferred that the array is produced as a random array comprised of nucleic acid binding partners that are capable of hybridizing to essentially all parts of the genome, preferably all parts of the genome that are expressed as mRNA. A preferred microarray comprises discrete fragments of essentially the complete DNA of the organism that is to be analyzed. An advantage of such an array is that the effect of a chemical compound may then also be detected on metabolic functions that have not yet been identified, i.e. for which no ORFs are known to exist in the genome of the test organism. An additional advantage of using micro-arrays with randomly generated nucleic acid fragments is that the method may also be performed with organisms of which the functional genome has not or only scarcely been investigated.

To make an array containing a genome-wide representation of a species of a micro-organism (e.g. *S. aureus*), a genomic library of one species or a mixed-genomic library of the organism can for instance be made by mixing genomic DNA (gDNA) of several (2-20, for instance 8 strains of the species (e.g. 8 *S. aureus* strains). The strains that are assembled in such a mixture may for

instance be selected on the basis of exhibiting different antibiotic resistance profiles (spectrum of sensitivity), preferably together covering most of the known types of antibiotic-resistance. , The strains may further for instance be selected on the basis of not having extensive amounts of plasmids (e.g. as may
5 be determined by agarose-gel analysis of isolated gDNA).

In order to prepare array containing a genome-wide representation of a species of a micro-organism the gDNA or gDNA mix can be sheared, for instance by sonication and the individual fragments may be separated, for instance by electrophoresis, e.g. on agarose gel. DNA-fragments of suitable size
10 (e.g. approx. 1-3 kb) may then be excised and isolated/purified (for instance via binding to glass-milk). The isolated DNA fragments may then be pretreated to facilitate efficient cloning (e.g. blunt-end cloning) into a suitable vector, e.g. a bacterial plasmid. The cloning will generally involve the ligation of the fragments into the vector and transformation of host cells, and the
15 regeneration and isolation of individual clones. Genomic inserts from each clone may then be used as genomic probe on a microarray. The probes may be obtained by PCR-amplification with vector-specific insert amplification primers, preferably using primers comprising an immobilizable group at one end for efficient spotting (e.g. on glass) of the various PCR amplification
20 products on the microarray.

A DNA microarray may be produced by methods known to the skilled person. The manufacture and use of DNA microarray for the detection of specific nucleic acid sequences is described abundantly (US 5,571,639; Sapolsky *et al.*, 1999, Genet. Anal.-Biomolecular Eng. 14, 187-192; Shena *et al.*, 1995, Science 270, 467-470; Sheldon *et al.*, 1993, Clinical Chem. 39, 718-
25 719; Fodor *et al.*, 1991, Science 251, 767-773).

The skilled person is capable of producing or acquiring arrays to his own design and the accompanying reading equipment from specilized suppliers (for instance Affymetrix Corp., Santa Clara, CA, USA for DNA arrays and
30 CIPHERGEN Biosystems, Fremont, CA, USA for ProteinChip Arrays).

By comparing the biochemical compositions before and after exposure to a chemical compound, and correcting for a general test influence with a control treatment, a biochemical response profile of that organism relating to that specific chemical compound may be obtained. A multitude of such biochemical response profiles may be stored in a database, so that the result of a measurement (a new biochemical response profile) may be easily compared to the available data. As a result, the database itself will grow in size and detail, making for better interpretations of forthcoming experiments.

By clustering some or all of the biochemical response profiles in the database with a newly acquired biochemical response profile, or by defining by computational analysis the mathematical relationship that defines a certain type of clustering of some or all of the biochemical response profiles in the database and then defining a mathematical formula that defines the position of the individual profiles in said type of clustering and applying that mathematical formula to the newly acquired biochemical response profile, one is able to determine the clustered position of said newly acquired biochemical response profile, amongst the known profiles. If said clustered position is within a known cluster, i.e. a cluster that comprises reference compounds of which the mode of action, target, cellular process affected, or any other classifiable characteristic is known, then one can assign that same characteristic to that compound with which the newly acquired biochemical response profile was obtained.

In order to strengthen this predictive character of the database or, in general, this classification scheme, the presence of the characteristic may be determined empirically.

If the clustered position of a newly acquired biochemical response profile is outside a known cluster, then one cannot assign a characteristic to that compound, yet the compound has potentially unknown characteristics, both intrinsically, and in terms of its effect on cells of organisms. Thus, such

compounds make for interesting objects of study and their effect on cells may be analysed in more detail.

In principle, any type of analysis may be performed to gain more insight in the target, mode of action or cellular process affected by such a compound.

5 The present invention now provides for one such a method wherein a mode of action of an antibiotic compound is analysed. The analysis starts with the determination of the clustered position in a classification scheme of the transcription response profile generated with said compound. In order to analyse a new mode of action said compound should generate a transcription
10 response profile whose clustered position is outside any known cluster of reference response profiles. In order to analyse an intrinsically known, but not yet analysed mode of action said compound should generate a transcription response profile whose clustered position is within a known cluster of reference response profiles. Irrespective of whether said clustered position corresponds
15 to a known cluster of reference response profiles or is outside any known cluster of reference response profiles, the transcription response profile indicates the changes that have occurred in the pool of messenger RNAs as a result of exposure of the test organism to the compound. Thus, the expression of some genes is affected as a result of the exposure and by determining the
20 spots on the micro-array that are substantially increased or decreased in intensity, the affected genes may be identified. Not all genes the expression of which is affected will be of equal relevance to the analysis of the mode of action of the compound. In stead, those genes that characterize the clustered position, which will be the genes that mainly determine the distinction between the
25 specific experimental condition and the control condition – such as can be identified by principle component analysis – are of major importance. If multiple genes involved in a specific process are affected, then by analysing the combination of genes affected, one can infer the cellular process affected by the antibiotic compound, a clustered position outside any known cluster indicating
30 a new process affected. If the analysis indicates that the expression of a

number of genes, involved in one cellular process, is changed under influence of the compound tested, this would point to said process as being affected by the compound. For instance genes involved in protein synthesis would indicate that the cellular process of protein synthesis is affected, indicating that the mode of action is interference with cellular protein synthesis. Another indicator will be genes that show a specific response towards one compound, but not to other treatments or compounds.

In yet a further aspect the present invention provides a method of identifying the molecular target of an antibiotic compound. The first step in such a method is the performance of a method of analysing a mode of action of an antibiotic compound as described above (clustered position within known clusters) or performing a method of identifying a new mode of action of an antibiotic compound as described above (clustered position outside known clusters). Such a method indicates the cellular process affected. Certain key enzymes or other cellular components that characterize said affected process are noted to form a group of potential molecular targets affected by the antibiotic compound. Subsequently, one can zoom in on that group, for instance by using antibody labels in a method of enzyme quantification, to identify the molecular target of the antibiotic compound.

The present invention also provides a method of identifying a new metabolic route. Essentially this method comprises the performance of a method of classifying a chemical compound as described above and determining for said compound that the clustered position is outside any known reference response profiles. As such, it is established that the cellular response to the compound was not seen before with other compounds. Then, the cellular process affected by said chemical compound is determined, essentially by using a method of identifying the new mode of action of said compound as described above. If said cellular process is a process which was not previously known to be affected, said process will involve a new metabolic route.

EXAMPLES

Example 1. Determination of antibiotic treatment by measuring the RNA expression of *Pseudomonas putida*

In the present test design, the *Pseudomonas putida* S12 strain was
5 exposed to high doses of different antibiotic compounds, each administered at a
concentration which is well beyond the MIC-value (Minimal Inhibitory
Concentration) known for such antibiotic compounds. The cells were fixed,
after which RNA was isolated from the cells. The isolated RNA was hybridized
on an array coated with genomic DNA fragments from *Pseudomonas putida*
10 S12. The data analysis involved normalization and analysis according to
principal component analysis (PCA). The test was carried out in duplicate and
repeated once.

Bacterial strain and growth conditions

15 *Pseudomonas putida* S12 pure culture was obtained by plating on
Trypton Soy Agar (TSA, Oxoid) followed by incubation at 30°C for 20 hours.
After this, the strain was grown in TSB for 1 day at 30°C in order to obtain a
pre-accumulation.

20 *Antibiotic treatment*

P. putida S12 cultures that are in a specific physiological state as a
result of antibiotic treatment were obtained by diluting an overnight
accumulation of *P. putida* S12 (35°C) 20 times in 50 ml fresh TSB medium in
Erlenmeyer flasks. In this manner, 4 simultaneous cultures of 50 ml were
25 produced that were allowed approximately 7 hours to grow to an OD₆₆₀ of
approximately 0.5 at 35°C. This was verified by measuring OD₆₆₀ of one culture
every 30 minutes. At the moment the OD₆₆₀ was 0.5, the two other cultures
were subjected to antibiotic treatment (compounds used in this experiment
were erythromycin, tetracycline, colistin and polymyxin B sulphate). The other

culture remained as control treatment. After 10 minutes, the cells from the three incubated cultures were quenched and harvested for RNA isolation.

Erythromycine and colistin were tested in several independent experiments.

5

RNA isolation

Quenching of metabolic activity in the culture was achieved by spraying the 10 ml culture in a stirred solution of a cold 60% methanol solution in water that was kept at -40°C (essentially as described by W. de Koning & K. van
10 Dam 1992. Anal. Biochem. 204:118-123). Then the cells were pelleted by 10-minute centrifugation at 4500 g at -20°C. The pellet was further treated at -45°C or stored at -80°C. RNA was essentially isolated as described by Chin-Yi & Wilkins (1994. Anal. Biochem. 223:7-12). A 2-ml screw-cap Eppendorf tube was filled with 0.4 grams of zirconium beads and 500 µl Trizol (Invitrogen,
15 GibcoBRL) at 0°C. The cell pellet (-45°C) was resuspended in 450 µl borate buffer (Chin-Yi & Wilkins, 1994) at 37°C and then added to the Eppendorf tube with beads and Trizol, mixed by hand and put on ice. The cells were broken up by beating them for 60 seconds in the Beadbeater (Biospec Products). The suspension was put on ice, after which 100 µl chloroform was
20 added, vortexed and then centrifuged at 12,000 g at room temperature. The water phase was extracted using 500 µl phenol/chloroform (1/1) and then using chloroform with interim centrifugation in order to obtain a pure water phase. To the water phase 0.4 volumes of isopropanol were added and 0.4 volumes of 0.8M NaCitrate, 1.2M NaCl, mixed and cooled on ice for 10 min. After that,
25 RNA was pelleted by centrifugation at 20,000 g at 4°C. The pellet was washed with 70% ethanol of -20°C and after centrifugation (20,000 g at 4°C) dried under vacuum. The pellet was dissolved in 100µl water.

Construction of microarray

To make genome-wide analysis of gene expression in *Pseudomonas putida* S12 possible, a genomic bank of this organism was made. For this purpose, 50 micrograms of genomic DNA were incorporated in TE buffer with 20% glycerol and sheared for 30 seconds at 1 bar in a nebulizer. The average
5 fragment size was estimated at 2-3 kb by means of gel electrophoresis. After precipitation, the ends were blunted in the presence of T4 DNA polymerase, Klenow polymerase and dNTPs. After heat inactivation of the enzymes, the ends were phosphorylated using T4 polynucleotide kinase. After heat
10 inactivation, the mixture was again separated on an agarose gel and fragments between 2 and 3 kb were cut from the gel and were purified using a Qiagen gel extraction kit. The fragments obtained in this manner were ligated in vector pSMART according to the "Clone Smart Blunt cloning kit" protocol (Lucigen Corp.). A part of this ligation mixture was transformed in *E. coli* ElectroMax DH10B cells (Invitrogen) and plated on ampicillin plates. Using a
15 colony picker, these colonies were picked and transferred into 96-well microtiter plates. After overnight growing at 37°C, glycerol was added to a final concentration of 15% and the glycerol stocks were stored at -80°C.

The genomic inserts from this bank were multiplied using PCR amplification in 96-well PCR plates. For this purpose, the following
20 components were put together: 5 microliters 10x SuperTaq buffer; 5 microliters 2 mM dNTP (Roche Diagnostics); 1 microliter primer L1; 1 microliter primer R1; 37 microliters MQ water; 1 microliter glycerol stock; 1.5 U SuperTaq polymerase. Primer L1 has the following base sequence: 5'-CAG TCC AGT TAC GCT GGA GTC -3'. Primer R1 has the following base sequence: 5'-CTT
25 TCT GCT ATG GAG GTC AGG TAT G -3'. Both primers contain a free NH₂ group at the 5'-end followed by a C6 linker. The following PCR program was used for amplification: 4 min at 94°C; 30x (30 sec at 94°C, 30 sec at 50°C, 3 min at 72°C); 10 min at 72°C; 4°C.

Following the reactions, the products were transferred to round-bottom
30 96-well plates and precipitated using isopropanol. The PCR products purified

in this manner were transferred to 384-well plates, in which the DNA, after evaporating to dryness, was incorporated in 10 microliters 3xSSC. These spot plates were used to transfer a total of 5500 PCR products to microarray slides on which they were spotted in a regular and ordered pattern. After spotting, these microarrays were blocked, after which they were ready for further use in hybridization experiments.

Labeling RNA

For the fluorescent labeling of total RNA, 12.5 micrograms of RNA were used. To this RNA, 0.5 microliters RNAsin, 1.25 micrograms of random primer (p(dN)6; Roche Diagnostics) and water were added to a final volume of 5 microliters. This mixture was incubated for 10 min at 70°C, then for 10 min at 20°C and then put on ice. Then the following components were added: 0.5 microliters RNAsin, 3 microliters 5X first strand buffer (SuperScriptII; Life Technologies), 1.5 microliters 0.1 M DTT, 3 microliters nucleotide mix (2.5 mM of dATP, dCTP, dGTP, 1 mM dTTP), 1 microliter Cy5-dUTP or Cy3-dUTP (Amersham Biosciences) and 1 microliter SuperScriptII enzyme (Life Technologies). After mixing, the reaction components were incubated for 2 hours at 42°C. Then the RNA was degraded by heating the samples for 2 min at 95°C, briefly spinning them down, adding 1.5 microliters fresh 2.5M NaOH and incubating this for 10 min at 56°C. By addition of 1.46 microliters 2.5M HAc, the reaction mixture is neutralized. This reaction mixture is purified by purification over an Autoseq G50 column (Amersham Biosciences) according to the accompanying directions. After this purification, 1/10 part of the labeled material is kept apart for spectrophotometric analysis.

Prehybridization / Hybridization / Washing

In preparation for the hybridization, slides were laid in Petri dishes in 20 ml prehybridization solution (1% BSA, 5x SSC, 0.1% SDS, filtered through a 0.45-micrometer-filter) and rotated for 45 minutes at 42°C. Then the slide

was dipped 5x in filter-sterilized milliQ water. Then the slide was washed again in pure milliQ water, after which the slide was dipped 5x in isopropanol. Then the slide was dried using a N₂ gun and was then ready for hybridization.

The samples labeled with Cy5-dUTP and with Cy3-dUTP that were
5 compared in one hybridization were combined in one cup. To this, 4 microliters yeast tRNA (25 micrograms / microliter) were added, after which the mixture was evaporated to dryness. After drying, the pellet was incorporated in 30 microliters EasyHyb (Roche Diagnostics) and denaturated (95°C, 1.5 min) after spinning down briefly. The mixture was then pipetted onto the prehybridized
10 slide, covered with a cover slip and introduced into a Corning Hybridization room and incubated overnight at 42°C.

After hybridization, the slide was removed from the hybridization room and directly introduced into 20 ml 1xSSC/0.2% SDS solution of 37°C. The slide was then transferred to a fresh 1xSSC/0.2% SDS solution of 37°C, the cover
15 slip staying behind in the first washing solution. After vigorous agitation, the slide was transferred to a 0.5xSSC washing solution of 37°C and again well agitated. Then the slide was transferred to a 0.2x SSC washing buffer and mixed for 10 min at 20°C on a rotation platform at 300 rpm. After replacing the washing buffer, this step was repeated. Then the slide was dried by
20 blowing away the residual liquid using a N₂ gun.

Scanning and image analysis

After washing, the slide was stored in the dark or directly used for scanning. A quick scan with a resolution of 30 micrometers led to a selection of
25 optimal scanning setting (ScanArray 4000, Perkin Elmer). The optimal scan of the Cy5 and Cy3 signal took place at a resolution of 10 micrometers and the resulting images were stored electronically. The images corresponding to a slide were opened in the software package ImaGene (version 4.2, BioDiscovery Inc.). After placing the grid, which represents the spot pattern of the DNA
30 samples on the slides, and spot recognition, signals and background were

calculated per spot. The data obtained were stored in electronic files and used for further data processing.

Data pre-processing

5 After calculation of M and A values (Roberts *et al.*, 2000, Science, 287, 873-880; Dudoit *et al.*, 2000, Statistics, UC Berkeley, Technical reports #578), the raw data were normalized using Loess normalization (Cleveland *et al.*, 1991, Statistics and Computing, volume 1 (1) pp. 47-62), leaving the following spots out of account: 1) spots with overload (with signal intensity in one or
10 both channels being greater than 64000); 2) spots with the background being greater than the signal; 3) reference spots; 4) spots that were assigned a different flag than 0 by the image analysis package.

 In this experiment, only spots that were found to have a good signal for all experiments were taken into account. After Loess normalization, the
15 individual array results were normalized to unity (sum of squares equal to 1).

Data analysis

 Then the dataset was analyzed using principal component analysis (PCA), with mean centering as the selected scaling method. Comparable
20 results can be obtained using other scaling methods or using other multivariate methods, whether or not using the information in which clusters the different samples are present.

Results

25 *P. putida* strain S12 was found to grow well or not in TSB depending on the amount and nature of the antibiotic used. For measuring cell response upon antibiotic treatment, an analysis of the gene expression pattern on the microarray after antibiotic treatment or control treatment was made. For this purpose, 10 minutes after antibiotic treatment, the cells were quenched,
30 harvested and further processed for microarray analysis.

Figure 4 shows a score plot in which the data from the set of experiments are depicted. The Figure shows a clear difference between the antibiotic treatments in the various experiments. The two axes represent the principal components that together describe a total of 72% of the total variance in the dataset.

Example 2. Determination of antibiotic treatment by measuring the RNA expression of *Staphylococcus aureus*

In the present test design, the *Staphylococcus aureus* ATCC6538 strain was exposed to high doses of different known and unknown antibiotic compounds, each administered at a concentration which is well higher than the MIC-value (Minimal Inhibitory Concentration) known for such antibiotic compounds. The cells were fixed, after which RNA was isolated from the cells. The isolated RNA was hybridized on an array coated with genomic DNA fragments from *Staphylococcus aureus* ATCC6538. The data analysis involved normalization and analysis according to principal component analysis (PCA). The test was carried out in duplicate and repeated once.

Bacterial strain and growth conditions

Staphylococcus aureus ATCC6538 pure culture was obtained by plating on Trypton Soy Agar (TSA, Oxoid) followed by incubation at 37°C for 20 hours. After this, the strain was grown in TSB for 1 day at 37°C in order to obtain a pre-accumulation.

Antibiotic treatment

S. aureus ATCC6538 cultures that are in a specific physiological state as a result of antibiotic treatment were obtained by diluting an overnight accumulation of *S. aureus* ATCC6538 (37°C) 100 times in 40 ml fresh TSB medium in Erlenmeyer flasks. In this manner, several simultaneous cultures of 40 ml were produced that were allowed approximately 3 hours to grow to an

OD₆₆₀ of approximately 0.5 at 37°C. This was verified by measuring OD₆₆₀ of one culture every 30 minutes. At the moment the OD₆₆₀ was 0.5, the other cultures were subjected to antibiotic treatment. Compounds used in this experiment were erythromycin, tetracyclin, chloramphenicol, trimethoprim, gentamycin, ceftazidin, actinomycin, rifampicin, ciprofloxacin, ofloxacin and nitrofurantion. Also two unknown growth inhibiting compounds, 'box9-e4' and 'box7-x3' were tested. All compounds were dissolved in DMSO short before use. Concentrations used for exposure were 2x MIC values for all compounds. Two cultures were used as control by adding pure DMSO. After 10 minutes, the cells from the incubated cultures were quenched and harvested for RNA isolation.

Ofloxacin, chloramphenicol, ceftazidin and nitrofurantion were tested in several independent experiments.

RNA isolation

Quenching of metabolic activity in the culture was achieved by spraying the 40 ml culture in a stirred 200 ml solution of 60% methanol / 66.7 mM HEPES pH 6.5 at -45°C (essentially as described by W. de Koning & K. van Dam 1992. Anal. Biochem. 204:118-123). The cells were pelleted by 20-minute centrifugation at 6000 x g at -20°C. The pellet was suspended in 8 ml methanol / HEPES solution at -45 °C and divided over 4 Eppendorf tubes. The pellets were further treated at -45°C or stored at -80°C. Cells in one of four Eppendorf tubes, were pelleted at 4 °C by a 5-minute centrifugation at 6000 g and suspended in 800 µl Trizol (Invitrogen, GibcoBRL) at 0 °C. This suspension was added to a 1.5-ml screw-cap Eppendorf tube, filled with 0.4 grams of 0.1 mm zirconium beads, mixed by hand and put on ice. The cells were broken up by beating them for 60 seconds at maximum speed in a Beadbeater (Biospec Products). The suspension was put on ice, after which 160 µl chloroform was added, vortexed and then centrifuged at 7000 x g at room temperature during 10 minutes. The water phase was transferred to a 2-ml Eppendorf tube and an

equal volume of 70% ethanol was added. After mixing, the RNA solution was applied on a RNeasy column (Qiagen) for further purification according to the Qiagen manual. The column was centrifuged at 8000 g, at 20 °C and 20 seconds and the flow-through was reloaded on the column. After centrifugation the flow-trough was discarded and 350 µl of RW1 buffer (Qiagen) was applied to the column. After centrifugation the bound RNA was subjected to DNase I treatment by adding a mixture of 10 µl DNase I stock solution and 70 µl RDD buffer (Qiagen) to the column and incubation at 37 °C for 15 minutes. 350 µl RW1 was applied again on the column and after centrifugation the column was washed twice with RPE buffer (Qiagen). To eliminate all traces of buffer, the column was centrifuged dry for 1 minute at 10.000 x g and the RNA was eluted by adding 30 µl of water to the column. After centrifugation at 10.000 x g for 1 minute this step was repeated and the purified RNA solution was ready for labeling. To determine the quality and amount of RNA, a sample was analysed on a BioAnalyzer (Agilent) or by gel electrophoresis.

Construction of S.aureus microarrays

To make an array containing a genome-wide representation of the species *S. aureus*, a mixed-genomic library of the organism was made by mixing gDNA of 8 *S. aureus* strains. Strains were selected that: (a) showed each a different profile of resistance to a broad set of antibiotics (together covering most types of antibiotic-resistance), and (b) did not contain a significant plasmid band in the agarose-gel analysis of their isolated gDNA. The gDNA mix was sheared by sonication (Branson sonifier 450) and ran in several lanes of a 0.8% agarose gel. DNA-fragments (approx. 1-3 kb) were excised and isolated via binding to glass-milk (Bio101-kit). The isolated fragments were pretreated with DNA-terminator End-repair kit (Lucigen Corp.) to facilitate efficient (blunt) cloning into bacterial plasmids (pSmartHCKan vector, CloneSmart Blunt Cloning Kit, Lucigen). Part of the ligationmix (1µl) was transformed to 25 µl *E.coli* cells (*E. coli* 10G supreme electrocompetent cells, Lucigen) by electroporation (0,1cm-

gap cuvettes Eurogentec, BioRad Pulsor, 25 uF, 2 ohms, 1,6 kV), regenerated in TB-culture medium, plated on TY-plates with 30 µg/ml kanamycin and grown overnight at 37°C. Using tooth-picks, colonies were transferred to into 96-well microtiterplates (32 plates, 150 µl/well TY medium with 30 µg/ml kanamycin). After overnight growing at 37°C, glycerol was added (final conc. 15%) and the glycerol-stocks were stored at -80°C.

The genomic inserts from each clone in the well-plates were multiplied using PCR-amplification in 96-well PCR-plates (22 plates). PCR reactions contained 50 µl reactionmix/well with 1xSuperTaq buffer, 0.2mM of each dNTP (Roche Diagnostics), 0.4 µM each of forward and reverse primer (vector-specific insert amplification primers), 1.5 U SuperTaq-DNA-polymerase and 1µl glycerol stock from corresponding well of gDNA-bank. Both primers contain a free NH₂-group for immobilization of the fragment coupled via a C6-linker to the 5' end of the primer. The following PCR-program was used: 4 min 94°C, 30x (30 sec 94°C, 30 sec 50°C, 3 min 72°C), 10 min 72°C and soaking at 4°C. Following the amplifications, the 50 µl PCR-products were transferred to 96-well round-bottom plates and precipitated by adding 150 µl NaAc/isopropanol mix (0.2M NaAc, 67% isopropanol final conc. each), incubation 1hr -80°C, spinning (1 hr, 2.5 krpm, 4°C), removal of supernatant and washing with 1 µl 70% ethanol. DNA-pellets were resuspended in 50 µl water/well, transferred to 384-well plates, dried (speed vac) and resuspended in 15 µl 3xSSC-buffer per well. Approximately 2100 different PCR-products (from 2100 different clones) were used for spotting the micro-arrays. The PCR-products were spotted in duplo on series of maximal 75 "aldehyde" coated slides (Cell Associates)) using an ESI micro-array printer in combination with 24 TeleChem Stealth micro spotting quill-pins (approx 100 µm diameter).

After spotting, slide surfaces were blocked by treatment at room temperature with boro-anhydride: 2x 5 min in 0.2% SDS, 2x 5min in water, 1x 2 min 100 °C water, 10 min in boro-anhydride buffer (1.7 g NaBH₄ in 510 ml PBS-buffer and 170 ml 1% ethanol), 3x 5 min in 0.2% SDS, 3x 5 min in water,

2 sec in 100°C water, dry with N₂ flow. PBS (phosphate buffered saline) is 6.75 mM Na₂HPO₄, 1.5 mM K₂HPO₄, 140 mM NaCl, 2.7 mM KCl pH 7.0. (1.2 g Na₂HPO₄, 0.2 g K₂HPO₄, 8.0 g NaCl, 0.2 g KCl per liter, pH 7.0).

5 *Labeling RNA*

For the fluorescent labeling of total RNA, 12.5 µg of dried RNA was used. To this RNA, 0.5 µl RNAsin (40 U/µl Promega), 5 micrograms of random primer (p(dN)₆; Roche Diagnostics) and water was added to a final volume of 5 µl. This mixture was incubated for 5 min at 70°C, then for 10 min at 20°C
10 and put on ice. The following components were added: 0.5 µl RNAsin, 2.5 µl 5x first strand buffer (SuperScriptII; Life Technologies), 1 µl 0.1 M DTT, 2 µl nucleotide mix (2.5 mM of dATP, dCTP, dGTP, 1 mM dTTP), 1 µl Cy5-dUTP or Cy3-dUTP (Amersham Biosciences) and 1 µl SuperScriptII enzyme (Life Technologies). After mixing, the reaction components were incubated for 2
15 hours at 42°C. The RNA was degraded by adding 2.5 µl fresh 2.5 M NaOH and incubating this for 15 min at 56°C. By addition of 2.5 µl of 2.5M HAc, the reaction mixture is neutralized. This reaction mixture was purified by using an Autoseq G50 column (Amersham Biosciences) according to the instructions of the manufacturer. After this purification, 1/10 part of the labelled material
20 was kept apart for spectrophotometric analysis.

Prehybridization / Hybridization / Washing

In preparation for the hybridization, slides were placed in Petri dishes in 20 ml prehybridization solution (1% BSA, 5x SSC, 0.1% SDS, filtered
25 through a 0.45-µm-filter) and rotated for 45 minutes at 42°C. After dipping once in a large volume of milliQ water, the slides were washed twice in 50 ml milliQ water, and dried using a N₂ gun.

The samples labelled with Cy5-dUTP and with Cy3-dUTP that were compared in one hybridization were combined in one cup. To this, 4 µl yeast
30 tRNA (25 µg / µl) was added, after which the mixture was evaporated to

dryness. After drying, the pellet was dissolved in 30 μ l EasyHyb (Roche Diagnostics), denaturated (95°C, 2 min) and briefly spun down. The mixture was then pipetted onto the prehybridized slide, covered with a cover slip, introduced into a Corning Hybridization Chamber and incubated overnight at 42°C.

After hybridization, the slide was removed from the hybridization chamber and directly introduced into 50 ml 1xSSC/0.2% SDS solution of 37°C. The slide was then transferred to a fresh 1xSSC/0.2% SDS solution of 37°C, the cover slip staying behind in the first washing solution. After vigorous agitation, the slide was transferred to a 0.5xSSC washing solution of 37°C and again well agitated. Then the slide was transferred to a 0.2x SSC washing buffer and mixed for 10 min at 20°C on a rotation platform at 300 rpm. After replacing the washing buffer, this step was repeated. Then the slide was dried by blowing away the residual liquid using a N₂ gun.

Scanning and image analysis, data pre-processing and data analysis were essentially as described in Example 1.

Results

S. aureus strain ATCC6538 was found to grow well or not in TSB depending on the amount and nature of the antibiotic used. For measuring cell response upon antibiotic treatment, an analysis of the gene expression pattern on the microarray after antibiotic treatment or control treatment was made. For this purpose, 10 minutes after antibiotic treatment, the cells were quenched, harvested and further processed for microarray analysis.

Figure 5 shows a score plot in which the data from the set of experiments are depicted. The Figure shows a clear difference between the antibiotic treatments in the various experiments. The two axes represent the principal components that together describe a total of 72% of the total variance in the dataset.

Claims

1. Method of producing a classification scheme for chemical compounds comprising clustering according to similarity the biochemical response profiles of an organism as determined by exposing said organism for a of period less than one generation time to a multitude of individual chemical compounds.
5
2. Method according to claim 1, wherein the metabolism of the organism is quenched directly after said exposing time.
3. Method according to claim 1 or 2, wherein said clustering according to
10 similarity comprises principal component analysis.
4. Method according to any one of the preceding claims, wherein said chemical compounds are not cytotoxic to mammalian cells.
- 15 5. Method according to any one of the preceding claims, wherein said organism is susceptible to said individual chemical compounds.
6. Method according to any one of the preceding claims, wherein said chemical compounds are antibiotic compounds.
20
7. Method according to any one of the preceding claims, wherein said multitude of individual chemical compounds comprises reference compounds with known mode of action and wherein said profiles are reference response profiles clustered according similarity in mode of action.
25
8. Classification scheme for chemical compounds obtainable by a method according to any one of claims 1 to 7.

9. Method of classifying a chemical compound, comprising the steps of:

- a) providing a classification scheme for chemical compounds by performing a method of any one of claims 1-6;
- b) exposing an organism to said chemical compound;
- c) determining the biochemical response profile of said organism, and
- d) determining in said scheme the clustered position of said biochemical response profile.

10. Method of classifying an antibiotic compound according to mode of action comprising the steps of:

- a) providing a classification scheme for chemical compounds by performing a method of any one of claims 1-6, wherein said chemical compound is an antibiotic compound, wherein said multitude of individual antibiotic compounds comprises reference compounds with known mode of action and wherein said profiles are reference response profiles clustered according similarity in mode of action;
- b) exposing an organism to said antibiotic compound;
- c) determining the biochemical response profile of said organism;
- d) determining in said scheme the clustered position of said biochemical response profile, and
- e) assigning to said antibiotic compound the mode of action of the corresponding cluster of reference response profiles or assigning to said antibiotic compound a new mode of action in case said position is outside any known cluster of reference response profiles.

11. Method of analysing a mode of action of an antibiotic compound comprising the steps of:

- a) performing a method of classifying an antibiotic compound according to claim 10, wherein the biochemical response profile is a transcription response profile, thereby determining for said antibiotic compound the clustered position of the transcription response profile;
- 5 b) identifying from said transcription response profile the genes of said organism of which the expression is substantially affected by exposure to said antibiotic compound and which characterize said clustered position, and
- 10 c) determining from the identity of said genes the cellular process affected by said antibiotic compound.
12. Method of identifying a new mode of action of an antibiotic compound comprising the steps of:
- a) performing a method of classifying an antibiotic compound according to claim 10, wherein the biochemical response profile is a transcription response profile;
- 15 b) selecting an antibiotic compound of which the corresponding transcription response profile has a clustered position outside any known cluster of reference response profiles;
- 20 c) identifying from the transcription response profile of said selected compound the genes of said organism of which the expression is substantially affected by exposure to said selected antibiotic compound and which characterize said clustered position, and
- 25 d) determining from the identity of said genes the cellular process affected by said selected antibiotic compound thereby identifying the new mode of action.
13. Method of identifying the molecular target of an antibiotic compound comprising the steps of:
- 30 a) performing a method according to claim 11 or 12;

- b) selecting a group of potential molecular targets characterizing said cellular process affected by said antibiotic compound, and
- c) identifying the molecular target of said antibiotic compound in said group.

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14. Method of identifying a new metabolic route comprising the steps of:

- a) performing the method according to claim 9, wherein the biochemical response profile is a transcription response profile, wherein said multitude of individual chemical compounds comprises reference compounds with known mode of action and wherein said profiles are

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- b) selecting a chemical compound of which the corresponding transcription response profile has a clustered position outside any known cluster of reference response profiles;

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- c) identifying from said transcription response profile the genes of said organism of which the expression is substantially affected by exposure to said chemical compound and which characterize said clustered position, and

20

- d) determining from the identity of said genes the cellular process affected by said chemical compound thereby identifying a new metabolic route.

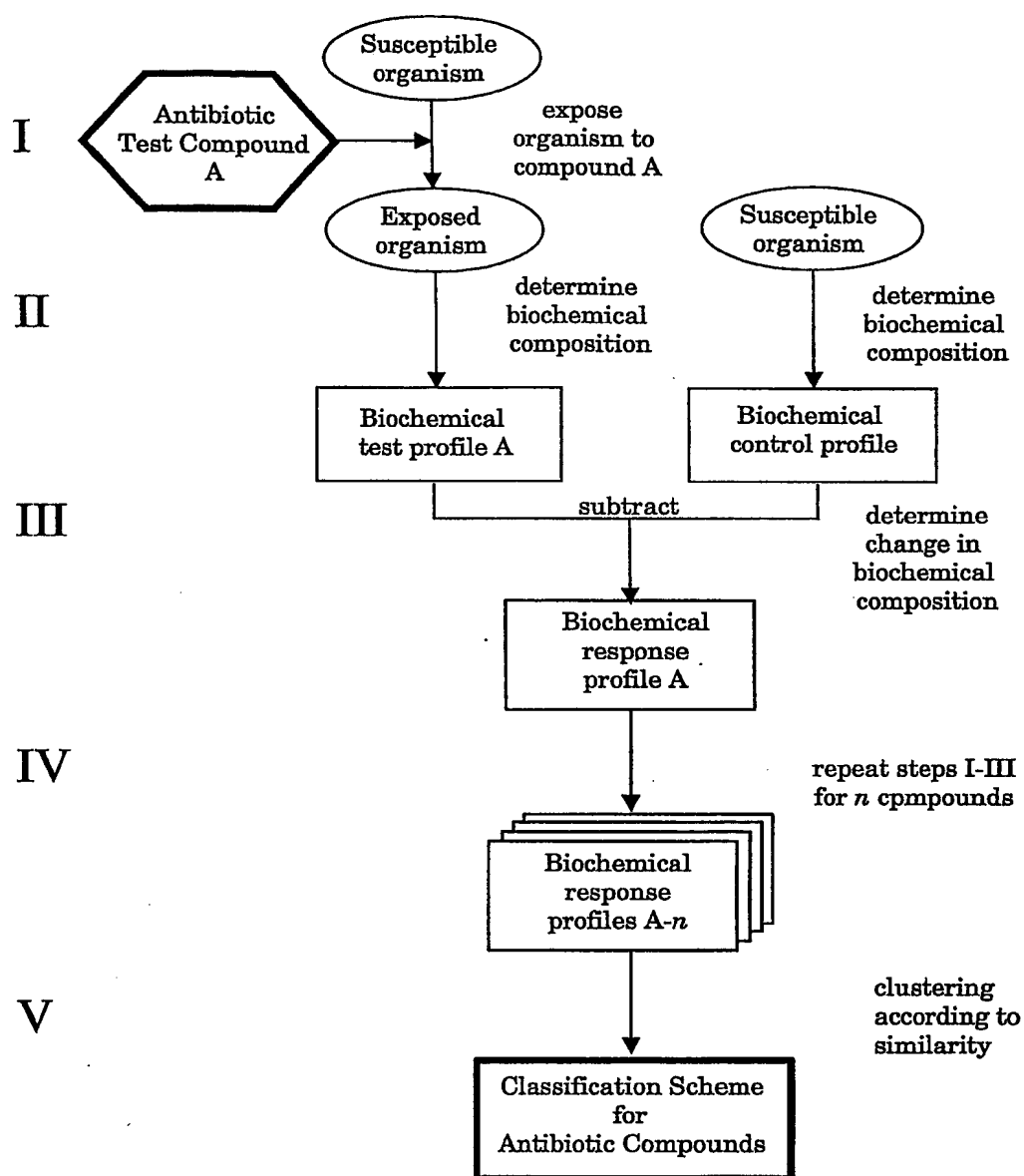


Figure 1

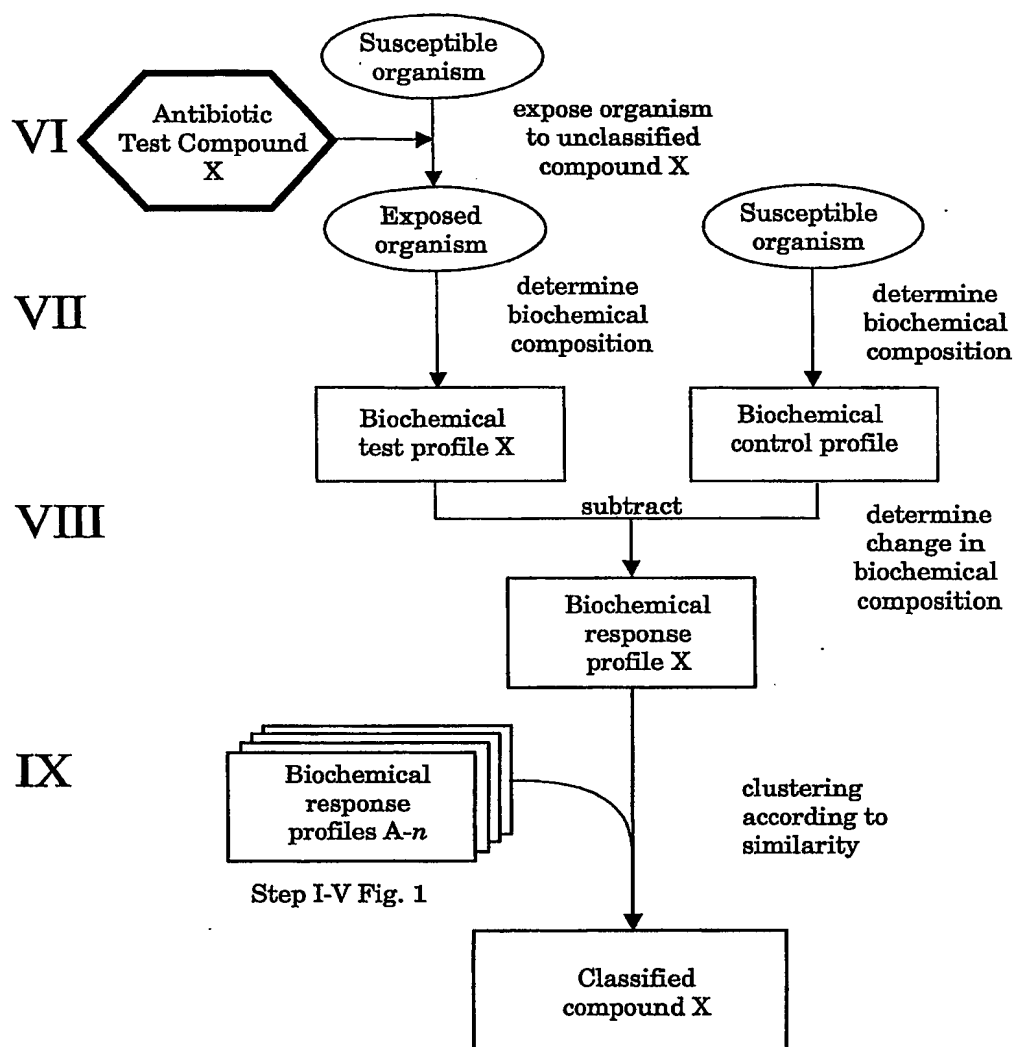


Figure 2

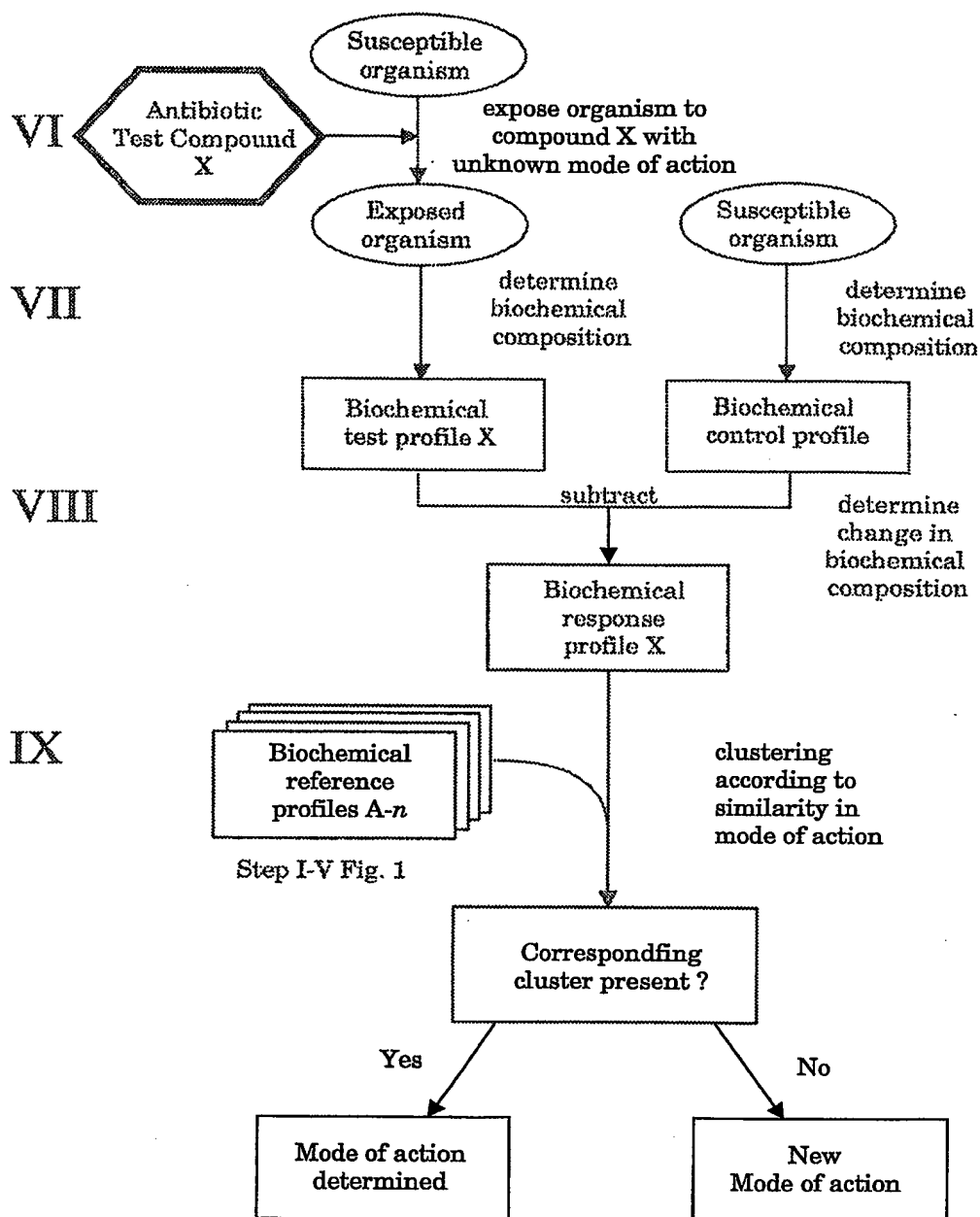


Figure 3

38 arrays Cy5/Cy3, PCA, mean centering

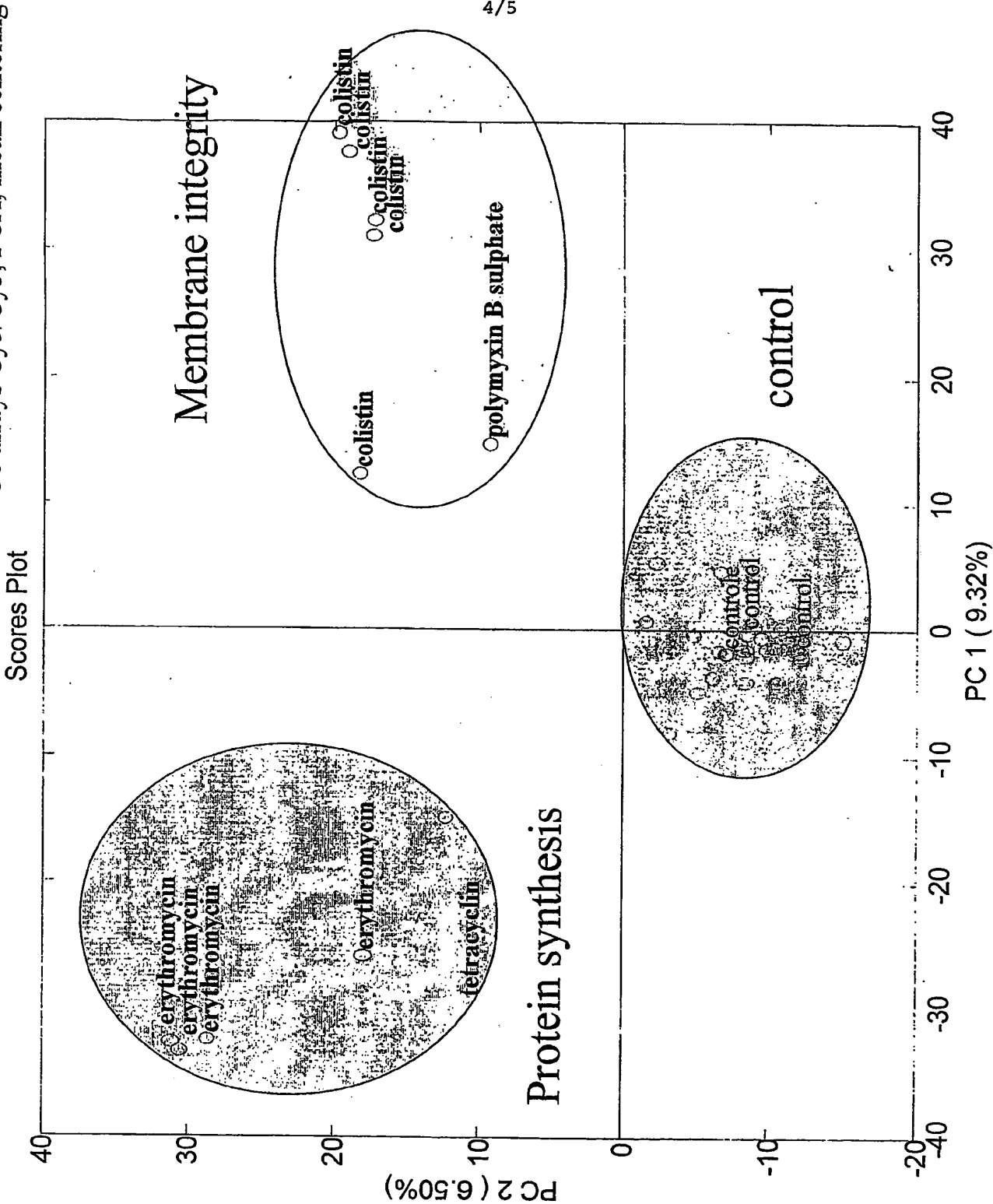


Figure 4

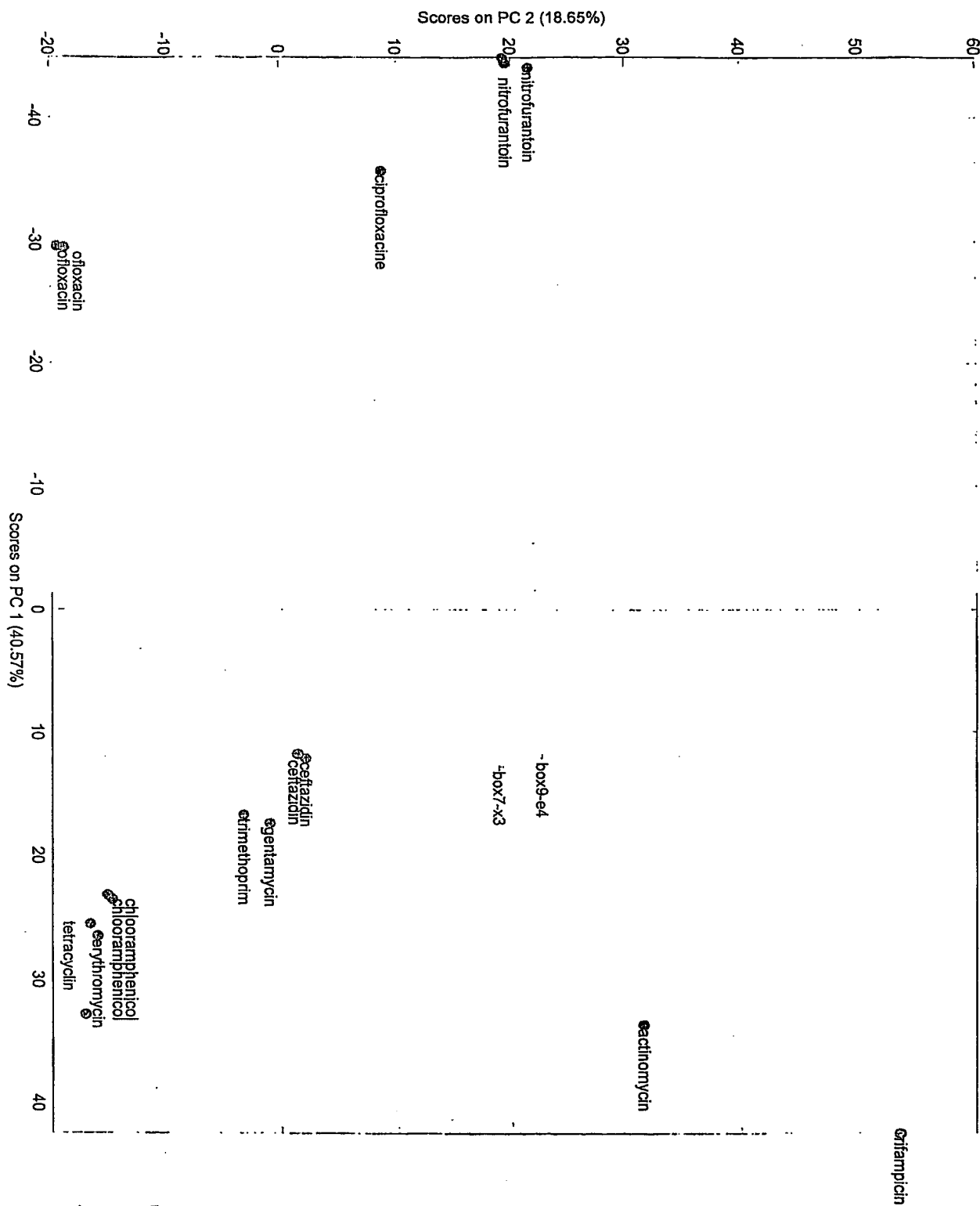


Figure 5.

INTERNATIONAL SEARCH REPORT

Application No
NL2004/000719

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/18 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, EMBASE, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>NG WAI-LEUNG ET AL: "Transcriptional regulation and signature patterns revealed by microarray analyses of Streptococcus pneumoniae R6 challenged with sublethal concentrations of translation inhibitors." JOURNAL OF BACTERIOLOGY, vol. 185, no. 1, January 2003 (2003-01), pages 359-370, XP002312588 ISSN: 0021-9193 abstract page 360, column 1, paragraph 3</p> <p style="text-align: center;">-/-</p>	1-7,9-14

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

5 January 2005

Date of mailing of the international search report

21/01/2005

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Vadot-Van Geldre, E

INTERNATIONAL SEARCH REPORT

Application No
/NL2004/000719

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>CHAN P F ET AL: "Microarray gene expression profiling to study the mode of action of DNA replication class of inhibitors in Staphylococcus aureus" ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR, vol. 102, 2002, page 9, XP008028290 102nd General Meeting of the American Society for Microbiology; Salt Lake City, UT, USA; May 19-23, 2002, 2002 ISSN: 1060-2011 abstract</p>	1,3-7, 9-14
X	<p>MOIR D T ET AL: "Microarray detection of gene expression profiles in Staphylococcus epidermidis" ABSTRACTS OF THE INTERSCIENCE CONFERENCE ON ANTIMICROBIAL AGENTS AND, vol. 41, 2001, page 249, XP008028401 41st Annual Meeting of the Interscience Conference on Antimicrobial Agents and Chemotherapy; Chicago, Illinois, USA; September 22-25, 2001, 2001 abstract</p>	1,3-7, 9-14
X	<p>WO 90/09096 A (HORVITZ HOWARD ROBERT ;CAMBRIDGE NEUROSCIENCE RES (US)) 23 August 1990 (1990-08-23) page 7, line 18 - page 9, line 10</p>	1,3-7, 9-14
X	<p>HAMADEH HISHAM K ET AL: "Gene expression analysis reveals chemical-specific profiles" TOXICOLOGICAL SCIENCES, vol. 67, no. 2, June 2002 (2002-06), pages 219-231, XP002253464 ISSN: 1096-6080 abstract page 225, column 2, paragraph 1; figures 2,3 page 228 - page 229</p>	1,3-7, 9-14
X	<p>HUGHES T R ET AL: "Functional discovery via a compendium of expression profiles" CELL, CELL PRESS, CAMBRIDGE, MA, US, vol. 102, no. 1, 7 July 2000 (2000-07-07), pages 109-126, XP002225641 ISSN: 0092-8674 page 115, column 2 - page 116, column 1 page 118, column 2, paragraph 1 page 124, column 1</p>	1,3-7, 9-14
	-/--	

INTERNATIONAL SEARCH REPORT

Application No
/NL2004/000719

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>ROBERTSON GREGORY T ET AL: "Global transcriptional analysis of clpP mutations of type 2 Streptococcus pneumoniae and their effects on physiology and virulence" JOURNAL OF BACTERIOLOGY, vol. 184, no. 13, July 2002 (2002-07), pages 3508-3520, XP002312589 ISSN: 0021-9193 abstract column 1 page 3509, column 2, paragraph 4 -----</p>	1-7,9-14

INTERNATIONAL SEARCH REPORT

application No.
PCT/NL2004/000719

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 8
because they relate to subject matter not required to be searched by this Authority, namely:
Article 52 (2)(d) EPC - Presentation of information
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Application No

/NL2004/000719

Patent document cited in search report		Publication date		Patent family member(s)		Publication date
WO 9009096	A	23-08-1990	AU	5106790 A		05-09-1990
			WO	9009096 A2		23-08-1990
<hr/>						